Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 317 (2008) 196–212
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Bullwhip neurons in the retina regulate the size and shape of the eye

Andy J. Fischer [a,*], Eric R. Ritchey [b], Melissa A. Scott [a], Angela Wynne [c]

[a] *Department of Neuroscience, College of Medicine, College of Medicine and Public Health, 4190 Graves Hall, 333 W. 10th Ave, Columbus, OH 43210-1239, USA*
[b] *College of Optometry, The Ohio State University, Columbus, OH, USA*
[c] *Neurosciences Graduate Studies Program, The Ohio State University, Columbus, OH, USA*

Received for publication 12 November 2007; revised 4 February 2008; accepted 8 February 2008
Available online 26 February 2008

Abstract

Bullwhip and mini-bullwhip cells are unconventional types of retinal neurons that utilize the neuropeptides glucagon, glucagon-like peptide 1 (GLP1) and substance P. These cells have been implicated in regulating the proliferation of neural progenitors in the circumferential marginal zone (CMZ) of the chicken retina. The purpose of this study was to investigate the roles of the bullwhip cells in regulating ocular size and shape. We found that intravitreal delivery of colchicine at postnatal day 7 destroys the vast majority (~98%) of the bullwhip and mini-bullwhip cells and their peptidergic terminals that are concentrated in the CMZ near the equator of the eye. Interestingly, colchicine-treatment resulted in excessive ocular growth that involved the expansion of equatorial diameter, but not axial length. Intraocular injections of glucagon completely prevented the equatorial expansion that occurs with colchicine-treatment. In eyes with undamaged retinas, exogenous glucagon suppressed equatorial eye growth, whereas glucagon receptor antagonists caused excessive equatorial growth. Furthermore, visual stimuli that increase or decrease rates of ocular growth caused a down- or up-regulation, respectively, of the immediate early gene Egr1 in the bullwhip cells; indicating that the activity of the bullwhip cells is regulated by growth-guiding visual cues. We found that the glucagon receptor was expressed by cells in the fibrous and cartilaginous sclera in equatorial regions of the eye. Taken together, these findings suggest that glucagon peptide released from the terminals of the bullwhip and mini-bullwhip cells regulates the growth of the equatorial sclera in a vision-dependent manner. Although the bullwhip and mini-bullwhip cells are not abundant, less than 1000 cells per retina, their influence on the development of the eye is substantial and includes vision-guided ocular growth.

Keywords: Retina; Glucagon; Eye growth; Neurons

Introduction

Achieving proper organ size is a fundamental process in developmental biology. For example, the eye must grow to a proper size to achieve clear vision. The combined refractive power of the lens and cornea must be precisely matched to axial length and equatorial circumference so that images are focused directly on the retina. If the eye grows too little (hyperopia; far-sightedness) or too much (myopia; near-sightedness) the images that are projected onto the retina tend to be blurred and visual acuity is poor. The overall growth of the eye is a function of the sclera, the connective-tissue sheath of the eye. Most vertebrates, including humans are born hyperopic, with their eyes too small (reviewed by Rada et al., 2006). However, as development proceeds, growth occurs so that the size and shape of the eye are precisely matched to the combined refractive power of the lens and cornea; this process is referred to as emmetropization (Rada et al., 2006; Wallman and Winawer, 2004). Visual experience is required to guide and "fine-tune" the growth of the eye, which requires retinal detection of image-blur (Wallman and Winawer, 2004). The neurons that detect image-blur and influence scleral growth remain unknown in the human retina, but may have been identified in the chicken. Glucagon-expressing amacrine cells (GACs) in the chicken retina are known to respond selectively to visual stimuli that slow or accelerate ocular growth by up- or down-regulating, respectively, the expression of the immediate early gene Egr1 (Fischer et al., 1999a). In addition, recent reports have demonstrated that intraocular injections of glucagon suppress the excessive axial ocular

* Corresponding author. Fax: +1 614 688 8742.
E-mail address: fischer.412@osu.edu (A.J. Fischer).

0012-1606/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2008.02.023

growth that results from form-deprivation or divergent lens-wear (Feldkaemper and Schaeffel, 2002; Vessey et al., 2005a,b). These studies have suggested that the GACs regulate the axial growth of the eye, but definitive studies are still required to demonstrate unambiguously that these cells are directly responsible for regulating eye growth.

In some classes of vertebrates the growth of the sclera is somehow coordinated with the growth of the retina. The post-embryonic growth of the retina is the function of a zone of stem cells that is located at the peripheral edge of the retina. Retinal stem cells have been characterized in many different species of vertebrates (reviewed by Del Rio-Tsonis and Tsonis, 2003; Fischer, 2005; Otteson and Hitchcock, 2003; Raymond and Hitchcock, 1997, 2000; Reh and Levine, 1998). In the eyes of cold-blooded vertebrates, it has been known for several decades that retinal neurogenesis persists throughout the life of the animal and arises from stem cells that are found at the peripheral edge of the retina in the circumferential marginal zone (CMZ). In recent years, retinal stem cells have been identified in the postnatal chicken eye in the CMZ (Fischer et al., 2002; Fischer and Reh, 2000) and further anterior in the non-pigmented epithelium of the ciliary body (Fischer and Reh, 2003). Secreted growth factors have been shown to influence the proliferation of progenitors in the chicken CMZ. Factors that stimulate the proliferation of CMZ progenitors include insulin, insulin-like growth factor I (IGF-I), epidermal growth factor (EGF) (Fischer et al., 2002; Fischer and Reh, 2000) and Sonic Hedgehog (Moshiri et al., 2005). Conversely, glucagon and glucagon-like peptide 1 (GLP1) suppress the proliferation of progenitors in the CMZ (Fischer et al., 2005). Neurites containing glucagon, GLP1 and substance P are densely clustered within the CMZ at the peripheral edge of the retina (Fischer et al., 2005, 2006). The glucagon/substance P-immunoreactive (-IR) neurites that are ramified in the CMZ originate from unusual types of axon-forming neurons that have been termed bullwhip and mini-bullwhip cells (Ehrlich et al., 1987; Fischer et al., 2006). The bullwhip cells have large cell bodies that are located among the amacrine cells in the proximal inner nuclear layer (INL). The bullwhip cells are found only in ventral regions of the retina, whereas the mini-bullwhip cells (a smaller version of the bullwhip cells) are found only in dorsal regions of the retina. There are only about 240 bullwhip cells and 600 mini-bullwhip cells per retina, and these cells are distinctly different from other types of retinal cells (Fischer et al., 2005, 2006). It has been proposed that the bullwhip and mini-bullwhip cells convey visual information to the CMZ to control the addition of newly generated cells to the edge of the retina (Fischer et al., 2005). This control is mediated by neuropeptides, glucagon and GLP1, that act in opposition to insulin (or insulin-like growth factors) to regulate precisely the proliferation of retinal progenitors (Fischer et al., 2005).

Here we report that the ablation of the bullwhip and mini-bullwhip cells, and their terminals at the peripheral edge of the retina, results in excessive growth around the equator of the eye, whereas growth along the longitudinal axis of the eye is unaffected. Furthermore, we find that exogenous glucagon slows equatorial eye growth, resulting in smaller eyes, whereas glucagon receptor antagonists accelerate eye growth, resulting in larger eyes. We also provide evidence that the bullwhip cells selectively respond to growth-slowing and growth-accelerating visual stimuli. Our findings indicate that a small population of retinal neurons (<1000 per eye) can have a profound influence on achieving proper organ size.

Materials and methods

Animals

The use of animals in these experiments was in accordance with the guidelines established by the National Institutes of Health and The Ohio State University. Newly hatched leghorn chickens (*Gallus gallus domesticus*) were obtained from the Department of Animal Sciences at The Ohio State University and kept on a cycle of 12 h light, 12 h dark (lights on at 7:00 am). Chicks were housed in a stainless steel brooder at about 30 °C and received water and Purina™ chick starter *ad libitum*.

Intraocular injections

Chickens were anesthetized by inhalation of 2.5% isoflurane in oxygen, as described previously (Fischer et al., 1998b, 1999a,b). Injections were made using a 25-μl Hamilton syringe and a 26-gauge needle with a beveled, curved tip. Penetration of the needle was consistently made into the dorsal quadrant of the vitreous chamber. In all experiments, 20 μl of vehicle containing the test compound was injected into the experimental (left) eye, and 20 μl of vehicle alone was injected into the control (right) eye. The vehicle was sterile saline containing bovine serum albumin, 50 μg/ml, as carrier, and 5-bromo-2′-deoxyuridine (BrdU; 100 μg/ml) to label proliferating cells. Test compounds included colchicine (50–500 ng per dose; Sigma-Aldrich), recombinant human glucagon (50–1000 ng per dose; Sigma-Aldrich), purified porcine glucagon-like peptide 1 (GLP1; 200 ng per dose; Sigma-Aldrich), glucagon receptor antagonists (des-His^1, Phe^6, Glu^9-glucagon-NH$_2$; 1000 ng per dose; Sigma-Aldrich) or (2*R*)-*N*-[4-({4-(1-cyclohexen-1-yl)[(3,5-dichloroanilino)carbonyl]anilino}methyl)benzoyl]-2-hydroxy-*b*-alanine (NNC 25-0926; 200–1000 ng per dose; Novo Nordisk), recombinant human substance P (200 ng per dose; Sigma-Aldrich), or apomorphine (200 ng per dose; Sigma-Aldrich).

Form-deprivation experiments

Chickens were form-deprived as described previously (Fischer et al., 1998a,c, 1999b). We performed 3 separate experiments that involved form-deprivation. Experiment 1: eight chickens at postnatal day 7 (P7) received injections of 500 ng of colchicine into both eyes, as described above. Five days later translucent plastic goggles were affixed, using gel Crazy-Glue™, to the feathers surrounding the left eyes. The goggles were worn from P12 to P18, and eyes were measured on P18. Experiment 2: eight chickens received goggles over the left eyes at P4, goggles were removed at P9, and 2 h after the removal both eyes were measured and retinas processed for immunolabeling. Experiment 3: eight chickens received goggles at P8 and 24 h later both eyes were processed for immunolabeling.

Reverse transcriptase PCR

Retina, choroid plus pigmented epithelium, and sclera from two P7 chickens were isolated and placed in Trizol Reagent (~50 mg tissue/700 μl Trizol; Invitrogen). Total RNA was isolated according to the Trizol protocol and resuspended in 50 μl RNAse free water. Genomic DNA was removed by using the *DNA FREE* kit provided by Ambion. cDNA was synthesized from mRNA by using Superscript™ III First Strand Synthesis System (Invitrogen) and oligo dT primers according to the manufacturer's protocol. Control reactions were performed using all components with the exception of the reverse transcriptase to exclude the possibility that primers were amplifying genomic DNA.

PCR primers were designed by using the web-based program Primer 3 from the Whitehead Institute for Biomedical Research (http://frodo.wi.mit.edu/cgi-

bin/primer/primer3_www.cgi). Primer sequences were as follows: glucagon receptor forward=5′ ACATCCACATGAACCTCTTCG 3′, glucagon receptor reverse=5′ TAGTCCGTGTAGCGCATCTG 3′, GLP1 receptor reverse=5′ TGCCTCCACTACTGATGCTG 3′, and GLP1 receptor forward=5′ AAGAATGCCTGTGGTGACAGT 3′. Predicted product sizes (in base pairs) were 620 (glucagon receptor), and 529 (GLP1 receptor). PCR reactions were performed by using standard protocols, Platinum™ Taq (Invitrogen) and an Eppendorf thermal cycler. PCR products were run on an agarose gel to verify the predicted product sizes, TOPO-cloned (as described below) and sequenced to verify the identity of the products.

TOPO cloning

PCR products were run on a 1.2% agarose gel, extracted and purified by using Qiagen's Qiaex II kit according to the manufacturer's instructions. TOPO cloning was performed using Invitrogen's TOPO TA™ Cloning Kit and the pCR-II vector according to manufacturer's instructions.

In situ hybridization

Standard procedures were used for *in situ* hybridization as described elsewhere (Fischer et al., 2002, 2004). In short, riboprobes were synthesized by using a kit provided by Roche and stored at −80 °C until use. The anterior segments of P7 eyes were dissected in RNAse-free Hanks Balanced Salt Solution (HBSS), fixed for 2 h at room temperature in 4% paraformaldehyde buffered in 0.1 M dibasic sodium phosphate, and embedded in OCT™. Cryosections were processed for *in situ* hybridization with digoxigenin-labeled RNA probes as described previously (Fischer et al., 2002, 2004). Negative controls included hybridization of sense riboprobes and post-hybridization washes in 0.2× SSC at 70 °C. Hybridization was detected by using Fab fragments to digoxygenin that were conjugated to alkaline phosphatase (anti-DIG-AP; Roche) plus 10% normal goat serum, 10 mM levamisole, and 10 mM glycine in MABT (0.05 M maleic acid buffer, 0.1% Tween-20). Ntiro-blue tetrazolium (NBT) and 5-Bromo-4-Chloro-3′ Indolyphosphate *p*-Toluidine (BCIP) in 0.1 M NaCl, 0.1 M Tris–HCl pH 9.5, 0.05 M $MgCl_2$ and 0.01% Tween-20 were used to precipitate chromophore from the anti-DIG-AP.

Fixation, sectioning and immunocytochemistry

Ocular tissues were fixed, sectioned and immunolabeled as described elsewhere (Fischer et al., 1998b; Fischer and Stell, 1999). In short, enucleated eyes were bisected across the equator and the gel vitreous removed from the posterior eye cup. Posterior and anterior segments were fixed (4% paraformaldehyde plus 3% sucrose in 0.1 M phosphate buffer, pH 7.4, 30 min at 20 °C), washed three times in PBS (phosphate-buffered saline; 0.05 M sodium phosphate, 195 mM NaCl, pH 7.4), cryoprotected in PBS plus 30% sucrose, immersed in embedding medium (OCT-compound; Tissue-Tek), and freeze-mounted onto sectioning blocks. Transverse sections of the retina, nominally 12 μm thick, were cut from different regions of the eye and mounted onto SuperFrost Plus™ slides (Fisher Scientific). Sections from control and treated eyes from the same individual were placed consecutively on each slide to ensure equal exposures to reagents. Sections were air-dried and stored at −20 °C until use. Retinal whole-mounts were processed for indirect immunofluorescence as described elsewhere (Fischer et al., 2005, 2006).

Sections were thawed, ringed with rubber cement, washed three times in PBS, covered with primary antibody solution (200 μl of antiserum diluted in PBS plus 5% normal goat serum, 0.2% Triton X-100, and 0.01% NaN_3), and incubated for about 24 h at 20 °C in a humidified chamber. The slides were washed three times in PBS, covered with secondary antibody solution, and incubated for at least 1 h at 20 °C in a humidified chamber. Finally, samples were washed three times in PBS, rubber cement removed from the slides, and coverglasses mounted on 4:1 (v:v) glycerol to water.

Working dilutions and sources of antibodies used in this study included the following. (i) Mouse anti-glucagon raised to full-length human glucagon was used at 1:400 (Dr. M. Gregor, University of Tübingen via the Center for Ulcer Research and Education, UCLA). (ii) Rabbit anti-GLP1 raised to amino acids 1–19 of human GLP1 was used at 1:400 (4660-1604; Biogenesis Ltd.). (iii) Rat anti-substance P raised to full-length of human substance P conjugated to bovine serum albumin was used at 1:400 (ab6338; Abcam). (iv) Mouse anti-tyrosine hydroxylase raised to recombinant, full-length quail tyrosine hydroxylase was used at 1:50 (αTH; Developmental Studies Hybridoma Bank). (v) Mouse anti-Brn3a raised to amino acids 186–224 of mouse and used at 1:200 (5A3.2; Chemicon). (vi) Mouse anti-neurofilament raised to bovine NF-M was used at 1:2000 (RMO270, Dr. V. Lee, University of Pennsylvania). (vii) Rabbit anti-cleaved caspase 3 (CC3) was raised to amino acids 163–175 of human caspase 3 and used at 1:1000 (AF835; R&D Systems). (viii) Goat anti-Egr1 raised to amino acids 282–433 of recombinant human Egr1 was used at 1:1000 (AF2818; R&D Systems). The amino acid sequence used to raise the Egr1 antiserum is 88.2% identical to the chicken sequence, and the antibody produced patterns of labeling that were identical to those reported previously (Fischer et al., 1999a). Secondary antibodies included donkey-anti-goat-Alexa488, goat-anti-rabbit-Alexa488, and goat-anti-mouse-Alexa488/568 (Molecular Probes Inc., Eugene, OR) diluted to 1:1000 in PBS plus 0.2% Triton X-100.

Photography, measurements, cell counts, and statistical analyses

Photomicrographs were taken by using a Leica DM5000B microscope equipped with epifluorescence and a 12 megapixel Leica DC500 digital camera. Confocal microscopy was done by using a Ziess LSM 510 meta. Images were optimized for color, brightness and contrast, and double-labeled images overlaid by using Adobe Photoshop™6.0. Cell counts were made from at least 5 different individuals, and means and standard errors calculated on each data set. Counts of the bullwhip cells were made for the entire retina. To avoid the possibility of region-specific differences within the retina, cell counts were consistently made from the same region of retina for each data set.

Measurements of eye size

The axial length of the eye was measured by using a 3.5 MHz A-scan ultrasound (Zeiss). Photographs of enucleated eyes were taken using a 6.1 megapixel Nikon D100 SLR camera. High resolution digital images (>50 pixels/mm) of enucleated eyes were measured by using Image Pro Plus 6.2 (Mediacybernetics). Measurements obtained using Image Pro Plus 6.2 were highly reproducible and had low levels of sampling error (±0.22%) at a resolution of 50 pixels/mm or greater.

Results

Colchicine destroys bullwhip and mini-bullwhip cells when applied at postnatal day 7

To assess the functions of the bullwhip and mini-bullwhip cells we sought to identify a method by which to ablate these cells. We tested whether the disruption of microtubules with colchicine influenced the survival of the bullwhip cells. Delivery of colchicine into the vitreous chamber of newly hatched (P0) chickens is known to destroy dopaminergic and glucagonergic amacrine cells, as well as most of the ganglion cells (Fischer et al., 1999b; Morgan, 1981). However, the ability of colchicine to destroy retinal ganglion cells is quickly reduced during the early postnatal period (Morgan, 1981). The affects of colchicine on the bullwhip and mini-bullwhip cells remain unknown. Since these cells produce axon-like processes that form numerous terminal arbors in the CMZ we hypothesized that the bullwhip and mini-bullwhip cells may derive retrograde trophic support from CMZ progenitors. Thus, the destruction of CMZ progenitors and/or disruption of retrograde transport may affect the survival of the bullwhip cells. In initial experiments, we

injected 500 ng of colchicine into the vitreous chamber of the eye at P2 and harvested retinas 11 days later. Compared to saline-treated retinas (Figs. 1a and b), nearly all of the bullwhip cells had been destroyed by colchicine-treatment (Figs. 1c, d and g). In addition to the destruction of the bullwhip cells, numerous ganglion cells and GACs were destroyed (Figs. 1c, d and h), as reported previously (Fischer et al., 1999b). By contrast, a single intraocular injection of 50 ng colchicine at P7 did not significantly compromise the survival of bullwhip cells and GACs (Fig. 1g). However, 250 ng of colchicine destroyed nearly 80% of the bullwhip cells (Fig. 1g), whereas the GACs were left unaffected (data not shown). Similarly, 500 ng of colchicine at P7 destroyed more that 95% of the bullwhip cells, whereas numbers of GACs were not significantly reduced (Figs. 1e–h). The distribution of GAC somata and dendrites was not influenced by 500 ng of colchicine in central or ventral regions of the retina, where the bullwhip cells are normally found (Figs. 1e and f). Similarly, delivery of 500 ng of colchicine into the vitreous chamber of P14 eyes resulted in the destruction of >95% of the bullwhip cells, whereas the GACs were unaffected (data not shown).

In dorsal–peripheral regions of the retina, we found that the vast majority of mini-bullwhip cells were destroyed by colchicine-treatment at P7. Similar to the bullwhip cells, the mini-bullwhip cells are immunoreactive for both glucagon and substance P (Fischer et al., 2006). The mini-bullwhip cells can be easily distinguished from the smaller, conventional substance P-expressing amacrine cells in whole-mount preparations of the retina (Fischer et al., 2006). Compared to saline-treated retinas, which contained numerous substance P-immunoreactive mini-bullwhip cells and a dense agglomeration of neurites in the CMZ (Figs. 2a and b), colchicine-treated retinas were depleted

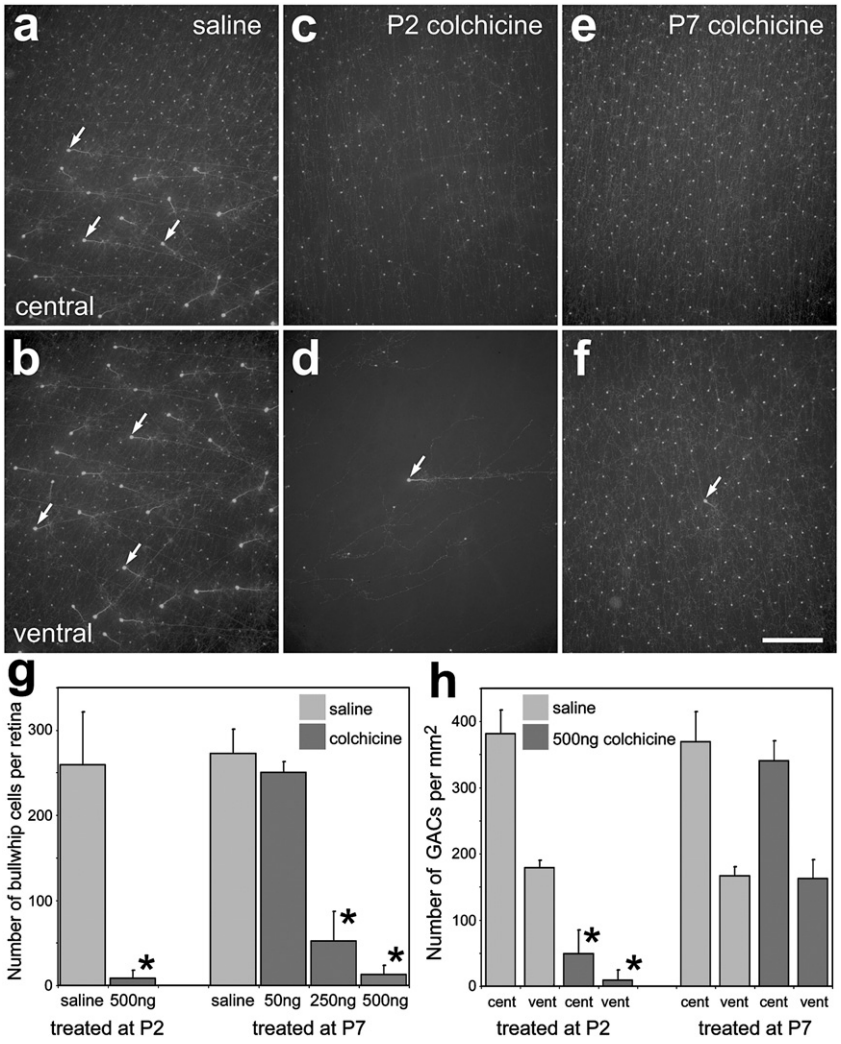

Fig. 1. Colchicine destroys the bullwhip cells when applied at postnatal day 7 (P7). Whole-mount preparations of the retina were labeled with antibodies to glucagon and photomicrographs obtained from central (a, c and e) and ventral (b, d and f) regions of the retina. Retinas were obtained from eyes that were treated with saline (a and b), 500 ng colchicine at P2 (c and d), or 500 ng colchicine at P7. The calibration bar (100 μm) in panel f applies to panels a–f. The arrows indicate representative bullwhip cells. Panel g is a histogram that shows the mean (±standard deviation) number of bullwhip cells per retina from eyes treated with saline or colchicine at P2 or P7. Panel h is a histogram that shows the mean (±standard deviation) number of glucagon-expressing amacrine cells (GACs) per mm^2 in retinas treated with saline or colchicine at P2 of P7. ANOVA was used to determine that there was significant ($p<0.0001$) difference among treatment groups and a paired Student's *t*-test was used to determine the significance of difference (*$p<0.0001$) between saline- and colchicine-treated data sets.

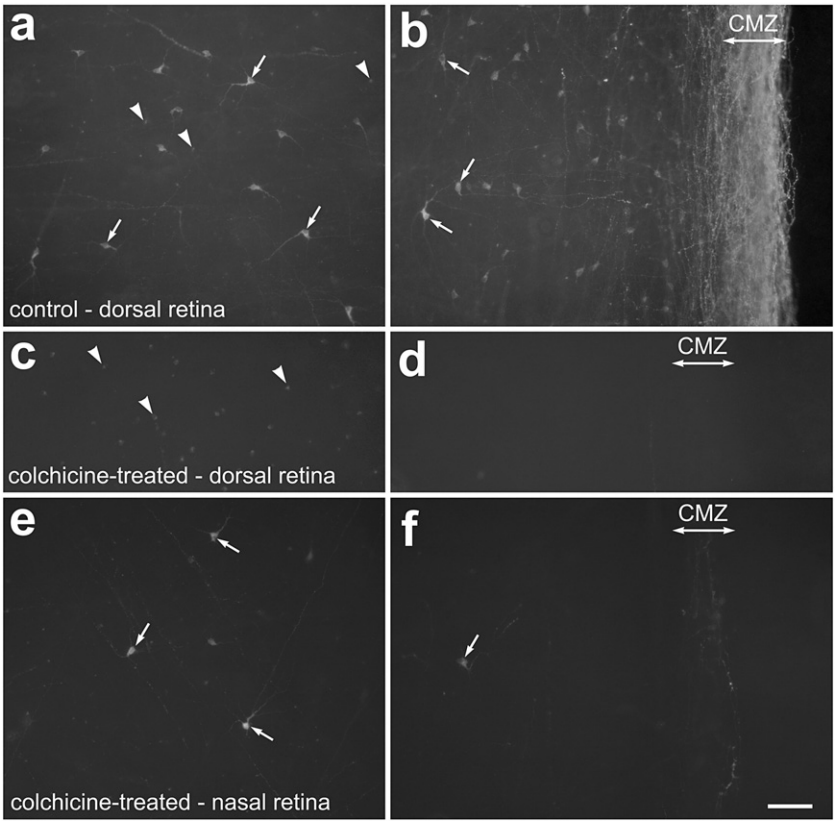

Fig. 2. The mini-bullwhip cells and their terminals in the CMZ are destroyed by colchicine-treatment. Whole-mount preparations of control (a and b) and colchicine-treated retinas (c–f) were labeled with antibodies to substance P. Images were made from peripheral (a, c and e) and far-peripheral (b, d and f) regions of the retina. Arrows indicate mini-bullwhip cells and arrow-heads indicate conventional substance P-immunoreactive amacrine cells. The calibration bar (50 μm) in panel f applies to all panels.

of mini-bullwhip cells, with the exception of a few (<10) cells in far-nasal regions of the retina (Figs. 2c–f). With the loss of the mini-bullwhip cells, we found a near-complete depletion of substance P-immunoreactive neurites in the CMZ (Figs. 2d and f). We occasionally found a few substance P-positive neurites in nasal regions of the CMZ, close to the somata of the mini-bullwhip cells that survived the colchicine-treatment (Fig. 2f).

To assess whether the glucagon-immunoreactive neurites that are clustered within the CMZ are depleted by colchicine we immunolabeled transverse sections of the far periphery of treated retinas. There was a near-complete absence of glucagon-immunoreactive neurites in peripheral regions of the retina and in the CMZ of colchicine-treated eyes (Figs. 3a–d). The glucagon-immunoreactive neurites that are normally clustered within the CMZ were ablated from all quadrants of the eye, with the exception of a few processes that could occasionally be detected in the nasal CMZ (Fig. 3d), consistent with observations of substance P-immunoreactivity in whole-mount preparations of colchicine-treated retinas. In addition, there was a significant depletion of dopaminergic neurites, which were immunoreactive for tyrosine hydroxylase (TH), in far-peripheral regions of the retina within 600 μm of the CMZ (Figs. 3e–h). Furthermore, there was a loss of ganglion cells that were immunoreactive for Brn3a (Figs. 3i–l) and neurofilament (Figs. 3m–p) in far-peripheral regions of colchicine-treated retinas. The loss of ganglion cells was more evident in temporal compared to nasal regions of the peripheral retina, and these losses were confined to within 700 μm of the CMZ.

To assess better whether colchicine ablated cells in addition to the bullwhip and mini-bullwhip cells, we labeled whole-mounts of treated retinas with antibodies to TH to identify the dopaminergic amacrine cells and Brn3a to identify the ganglion cells. The distribution of cell bodies and dendrites of the dopaminergic amacrine cells was not affected by colchicine-treatment in any regions of the retina (Supplemental Figs. 1a–b), with the exception of the far periphery (see Figs. 3f and h). Ventral and central regions of colchicine-treated retinas contained normal numbers of Brn3a-immunoreactive ganglion cells (Supplemental Figs. 1c–j). By comparison, colchicine destroyed numerous Brn3a-positive ganglion cells in dorsal regions of the retina (Supplemental Figs. 1c–j). Consistent with these findings, we found that there was a depletion of DRAQ5-labeled nuclei in the ganglion cell layer (GCL) in dorsal and far-peripheral regions of colchicine-treated retinas (Supplemental Fig. 2a). Similarly, in dorsal regions of colchicine-treated retinas there was a depletion of ganglion cells that were immunoreactive for calretinin, Islet1, and RA4 (Supplemental Figs. 2b–e). However, we failed to find evidence for the depletion of amacrine cells (Islet1, calretinin, calbindin, TH), bipolar cells (Islet1), horizontal cells (Islet1), or cones (calbindin, red–green opsin) in dorsal regions of colchicine-treated retinas. For all

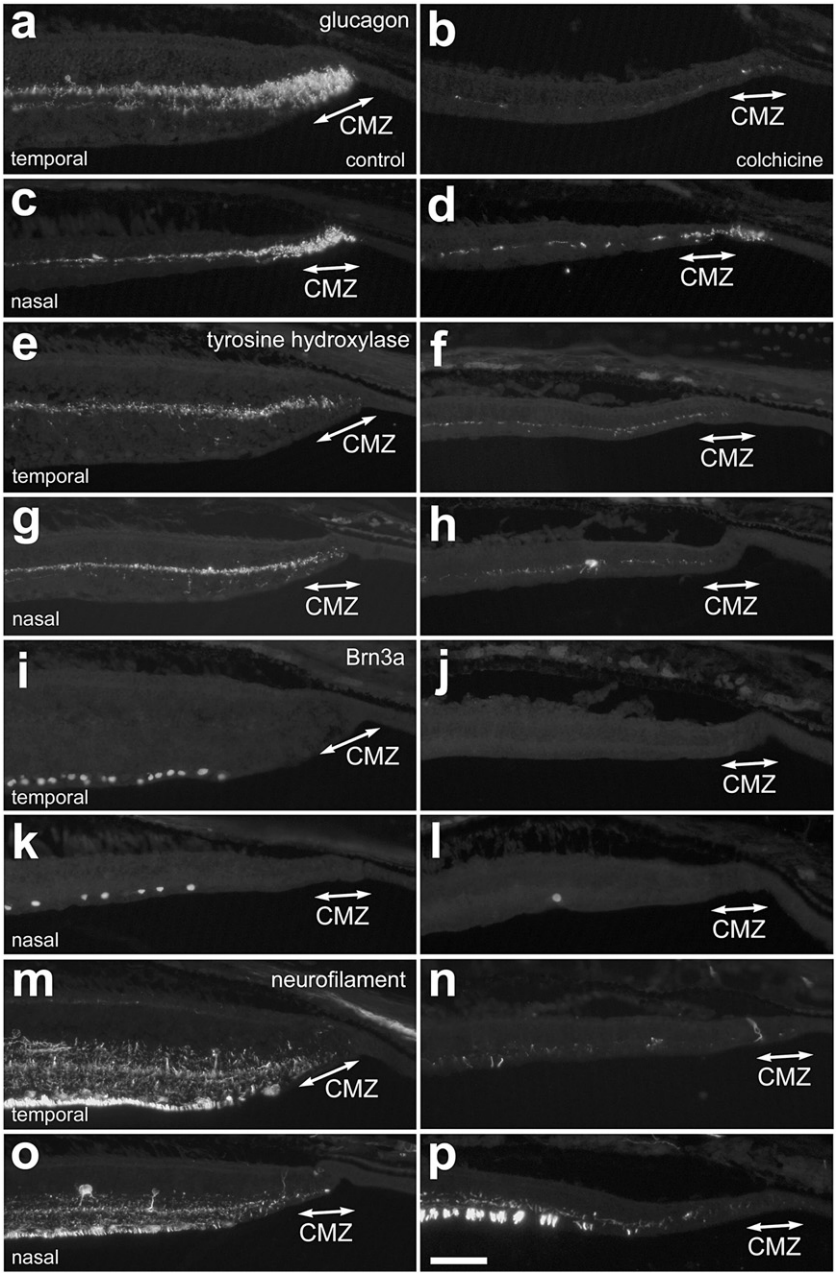

Fig. 3. Colchicine-treatment depletes the glucagon-immunoreactive neurites that are clustered within the CMZ, and destroys some retinal neurons in temporal regions of the far-peripheral retina. Vertical sections of the retina were labeled with antibodies to glucagon (a–d), tyrosine hydroxylase (e–h), Brn3a (i–l) or neurofilament (m–p). Double-ended arrows indicate the extent of the CMZ at the peripheral edge of the retina. The calibration bar (50 μm) in panel p applies to all panels. Abbreviation: CMZ—circumferential marginal zone.

markers, the labeling of sections of ventral regions of colchicine-treated retinas did not differ significantly from that in control retinas (data not shown).

Our findings indicate that the survival of retinal neurons (with the exception of the bullwhip cells) in central and ventral regions of the retina is not compromised by colchicine-treatment. These findings are consistent with previous reports that the retinal ganglion cells are most sensitive to colchicine during the first few postnatal days (Fischer et al., 1999b; Morgan, 1981). Our data indicate that the ganglion cells in dorsal and far-peripheral regions of the retina are destroyed by colchicine-treatment at P7.

Colchicine induces the apoptosis of retinal cells within 4 days after treatment

To characterize better the retinal damage caused by colchicine-treatment at P7 we applied antibodies to cleaved caspase 3 (CC3), to identify apoptotic cells, and the TUNEL method to identify dying cells that contained fragmented DNA. In untreated retinas we failed to find cells that were immunoreactive for CC3 (Fig. 4a). At 2 days after a single intraocular injection of colchicine, we found a few scattered CC3-immunoreactive cells in dorsal regions of the GCL (not shown). By comparison, there were numerous CC3-immunoreactive cells in far-peripheral

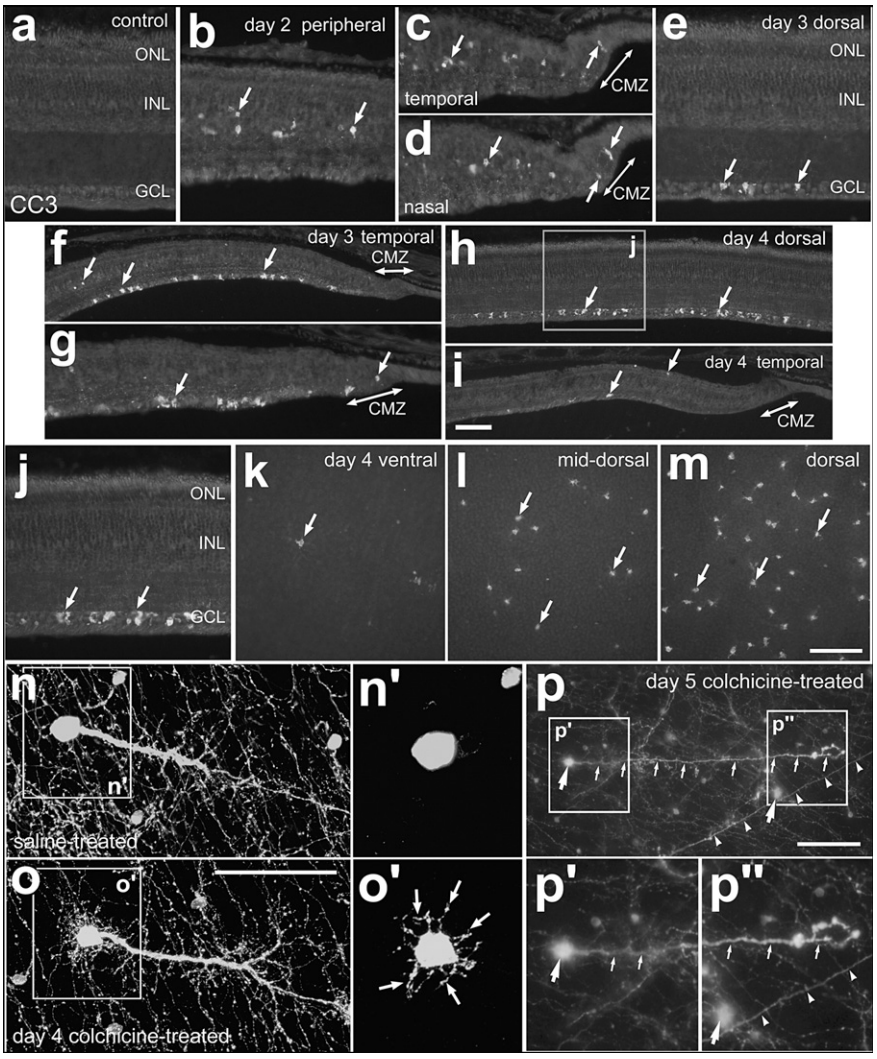

Fig. 4. Colchicine-treatment at postnatal day 7 results in apoptosis of retinal neurons and progenitors in the CMZ. Tissues were obtained from saline-treated (control) eyes (a and n), or at 2 (b–d), 3 (f–g), 4 days (h–m and o), or 5 days (p) after colchicine-treatment. Vertical sections were obtained from dorsal–central, nasal–peripheral or temporal–peripheral regions of the retina. Sections were labeled with antibodies to cleaved caspase 3 (CC3; a–m) and glucagon (n–p). Arrows indicated CC3-positive cells within the retina or CMZ (b–m), sprouted neurites (o′), or bullwhip cells that are degenerating (o and p). Double-ended arrows indicate the extent of the CMZ at the peripheral edge of the retina. The calibration bar (50 μm) in panel i applies to panels f–i, the bar in panel m applies to panels a–e and panels j–m, the bar in panel o applies to panels n and o, and the bar in panel p applies to panel p alone. Abbreviations: CC3—cleaved caspase 3, CMZ—circumferential marginal zone, ONL—outer nuclear layer, INL—inner nuclear layer, IPL—inner plexiform layer, GCL—ganglion cell layer.

regions of the INL (Figs. 4b–d). We consistently observed CC3-immunoreactive cells in the CMZ in both temporal and nasal regions of the retina (Figs. 4c and d). At 3 days after colchicine-treatment, increased numbers of CC3-immunoreactive cells were found in the GCL of dorsal regions of the retina (Fig. 4e), in the GCL in far-peripheral regions of the retina, and in the CMZ (Figs. 4f and g). By comparison, CC3-positive cells were not found in ventral or far nasal regions of the retina at 3 days after treatment (not shown). At 4 days after colchicine-treatment, numerous CC3-positive cells were observed in the GCL in dorsal regions of the retina (Figs. 4h, l and m), whereas few CC3-positive cells were observed in ventral (Fig. 4k) and peripheral regions of the retina (Fig. 4i). At 5 days after treatment, few CC3-positive cells were observed in dorsal or peripheral regions of the retina (data not shown). To corroborate the findings of the immunolabeling for CC3, we used the TUNEL method to identify nuclei that contained fragmented DNA. Our findings with TUNEL closely matched those obtained by labeling for CC3. We found abundant TUNEL-positive cells within the CMZ, in far-peripheral regions of the INL, and in dorsal regions of the GCL between 2 and 4 days after colchicine-treatment (Supplemental Fig. 3).

We failed to find significant numbers of bullwhip and mini-bullwhip cells that were CC3-positive (data not shown). However, we found numerous bullwhip cells that appeared to be degenerating in colchicine-treated retinas. At 4 days after treatment, most of the bullwhip cells sprouted numerous short processes that extended away from the cell bodies (Figs. 4n and o). By 5 days after treatment, most bullwhip cells had diminished glucagon-immunoreactivity within their primary neurites. In addition, the axons were fragmented, or truncated, and appeared

thickened or contained many varicosities (Fig. 4p). By 6 days after treatment, most bullwhip cells were no longer detectable (data not shown).

In summary, colchicine-treatment at P7 destroyed the bullwhip and mini-bullwhip cells, progenitors in the CMZ, some neurons in far peripheral regions of the retina, and some ganglion cells in dorsal regions of the retina.

Colchicine-treatment at P7 results in increased equatorial eye growth

We have reported previously that glucagon peptide is produced by the bullwhip cells, released from terminals clustered in the CMZ, and acts to suppress the proliferation of retinal progenitors (Fischer et al., 2005). Thus, colchicine-mediated ablation of glucagon-positive neurites in the retinal periphery might be expected to increase the proliferation of retinal progenitors in the CMZ. To test this hypothesis, we made 5 consecutive daily injections of BrdU (to label newly generated cells) into the vitreous chamber of colchicine-treated eyes starting at 7 days after treatment. We found that the ablation of glucagon-containing neurites within the CMZ did not increase numbers of BrdU-labeled cells within far peripheral regions of the retina (data not shown). This likely occurred because the colchicine destroyed significant numbers of progenitors in the CMZ (see Fig. 4 and Supplemental Fig. 3), and thereby reduced numbers of newly generated, BrdU-labeled cells that are added to the edge of the retina.

We have reported previously that colchicine-treatment on the day of hatching results in increased rates of ocular growth and axial elongation (Fischer et al., 1999b). Thus, we tested whether colchicine-treatment at P7 influences the growth of the eye. Unlike colchicine-treatment at P0, colchicine-treatment at P7 did not result in a significant increase in axial length; the distance from anterior cornea to posterior sclera (Figs. 5a, b and e). However, the equatorial diameter and circumference of colchicine-treated eyes were significantly larger than those in contralateral control eyes (Figs. 5c, d and e). In contrast, the corneal circumference was significantly smaller after colchicine-treatment (Fig. 5e). The corneal radii of curvature in control and colchicine-treated eyes were not significantly different (data not shown).

The ablation of glucagon from the bullwhip and mini-bullwhip cells may have resulted in increased rates of equatorial growth in colchicine-treated eyes. To test this hypothesis we injected both eyes with 500 ng colchicine at P7, and followed this with daily injections of 50 ng of glucagon or vehicle at P12 through P17 and measured eye size at P18. There was no significant difference in the axial length of colchicine-treated eyes that received injections of saline compared to length of eyes that received glucagon (Fig. 6a). By contrast, 50 ng doses of glucagon significantly prevented the increases in equatorial diameter and circumference resulting from colchicine-treatment (Fig. 6a). Colchicine-treated eyes that received injections of glucagon were uniformly narrower (across both nasotemporal and dorsoventral planes) than eyes treated with colchicine followed by injections of saline (Fig. 6a). The 50 ng injections of glucagon did not significantly affect colchicine-induced decreases in corneal circumference.

At higher doses, glucagon completely prevented colchicine-induced changes in ocular growth. Colchicine-damaged eyes treated with 6 consecutive daily injections of 500 ng glucagon were about 1 mm narrower in both the nasotemporal and dorsoventral equatorial diameters compared to eyes that were treated with colchicine and saline (Fig. 6b). This translated to a 3 mm decrease in the equatorial circumference (Fig. 6b). The 500 ng doses of glucagon did not influence the axial length of colchicine-treated eyes, whereas the circumference of the corneas of glucagon/colchicine-treated eyes was significantly larger compared to that of saline/colchicine-treated eyes (Fig. 6b). The growth-suppressing effects of 50 ng doses of glucagon were not as pronounced as those observed with 500 ng glucagon; the 50 ng-effect was about 30% as large as the 500 ng-effect (compare Figs. 6a and b). Interestingly, the equatorial circumference of eyes treated with colchicine and 500 ng glucagon was significantly ($p=0.026$; $n=6$) smaller than that of untreated eyes; 40.71 ± 0.87 mm versus 42.24 ± 1.18 mm, respectively. By comparison, the corneal circumference in eyes treated with colchicine and 500 ng glucagon was not significantly different ($p=0.78$; $n=6$) than that of untreated eyes; 18.36 ± 0.60 mm versus 18.47 ± 0.68 mm, respectively.

Since the bullwhip cells, mini-bullwhip cells, and their neurites within the CMZ are immunoreactive for GLP1 and substance P (Fischer et al., 2005, 2006), either GLP1 or substance P (or both) could act in parallel to glucagon to regulate equatorial eye growth. Accordingly, we made consecutive daily intraocular injections of 200 ng of GLP1 or 200 ng of substance P into colchicine-treated eyes. We found that the growth of eyes treated with colchicine was not significantly affected by either peptide (Figs. 6c and d).

Stone et al. (1989) reported that levels of retinal dopamine are influenced by form-deprivation and that a dopamine receptor agonist, apomorphine, inhibits the excessive ocular growth that occurs with form-deprivation. Since dopaminergic cells were depleted in far-peripheral regions of colchicine-treated eyes (see Figs. 3e–h), it is possible that excessive equatorial growth resulted from the depletion of dopamine near the equator. Thus, we tested whether 6 consecutive daily intraocular injections of 200 ng apomorphine inhibited colchicine-induced expansion of the equator of the eye. This dose of apomorphine has been shown to effectively inhibit form-deprivation-induced axial elongation (Rohrer et al., 1993; Stone et al., 1989). We found that the equatorial diameter and circumference of eyes treated with colchicine and apomorphine were not significantly different from those of the contralateral eyes that were treated with colchicine and saline (Fig. 6e). However, apomorphine caused a significant decrease in the axial length of colchicine-treated eyes (Fig. 6e). These findings suggest that the activation of dopamine receptors does not prevent colchicine-induced equatorial eye growth, and that the dopaminergic cells in far-peripheral regions of the retina are not responsible for regulating equatorial eye size.

We have reported previously that colchicine-treatment at P0 destroys the retinal circuitry that promotes the excessive ocular

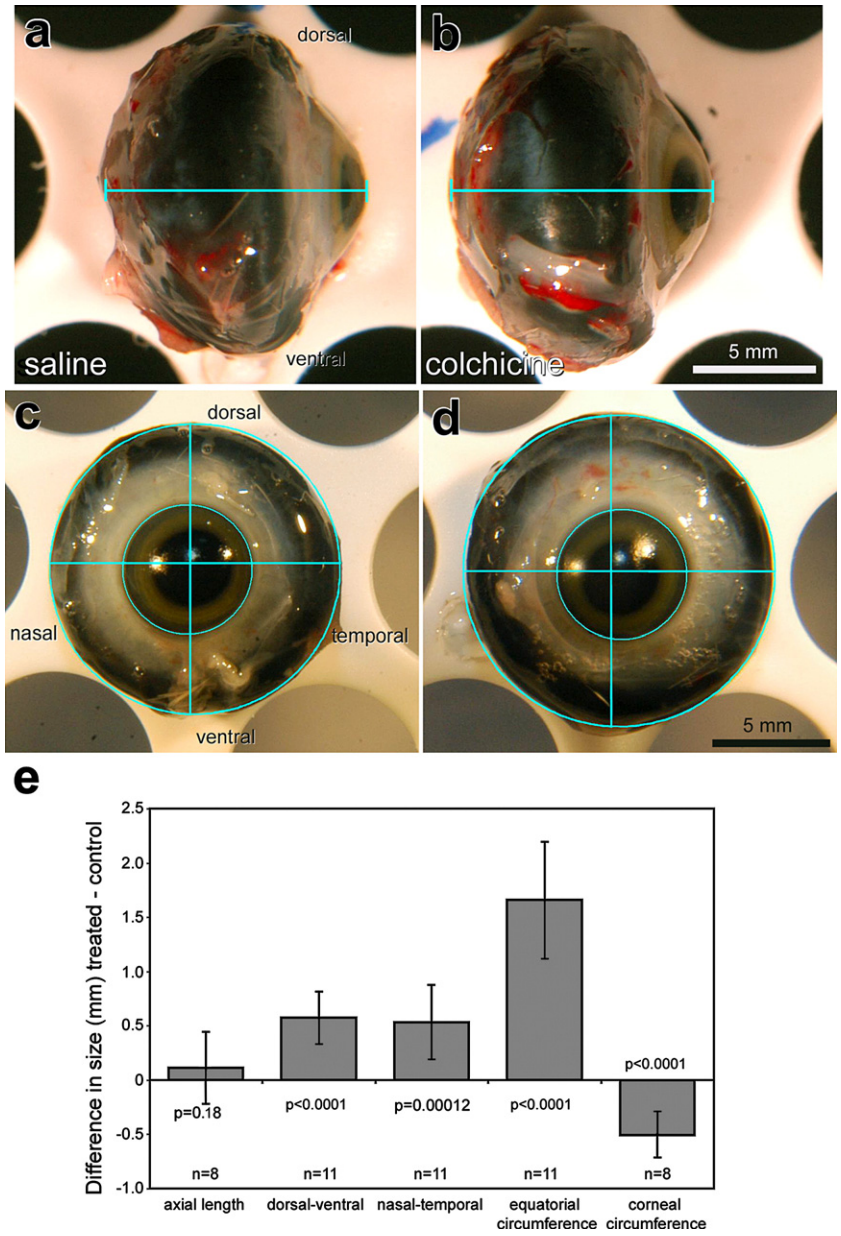

Fig. 5. Colchicine-treatment at P7 causes excessive growth around the equator of the eye, whereas there is no change in axial length. Eyes received a single intraocular injection of colchicine at P7 and were measured at P18. Photographs of eyes were taken on axis (c and d) or across the plane of the equator (a and b). The calibration bar (5 mm) in panel b applies to panels a and b, and the bar in panel d applies to panels c and d. The histogram in panel e illustrates the mean (±standard deviation) difference (treated minus control; mm) for the axial length, nasotemporal diameter, dorsal–ventral diameter, equatorial circumference, and corneal circumference of colchicine-treated eyes. The significance of difference (*p*-values) was determined by using a two-tailed, paired Student's *t*-test.

growth caused by form-deprivation (Fischer et al., 1999b). This may occur because P0 colchicine-treatment disrupts the retinal cells and circuitry, namely the GACs, that regulate vision-guided eye growth. Since P7 colchicine-treatment does not destroy the GACs (see Fig. 1), we hypothesized that form-deprivation would further increase rates of ocular growth in colchicine-treated eyes. We found that form-deprived/colchicine-treated eyes were significantly larger in axial length and equatorial dimensions compared to contra-lateral eyes that were treated with colchicine alone (Fig. 6f). However, form-deprivation failed to influence corneal circumference in the colchicine-treated eyes (Fig. 6f).

Glucagon and glucagon receptor antagonists influence the equatorial growth of eyes with undamaged retinas

To assess whether glucagon influences the normal growth of the eye, we made consecutive daily intraocular injections of 1 μg of glucagon from P6 through P11 and measured eyes at P12. We found that axial length was unaffected by glucagon-treatment (Fig. 7a). By contrast, the diameters across the naso-temporal and dorsoventral equator of glucagon-treated eyes were uniformly smaller, by about 0.5 mm, compared to the diameters of control eyes. Accordingly, the equatorial circumference of glucagon-treated eyes was >1 mm less than that of

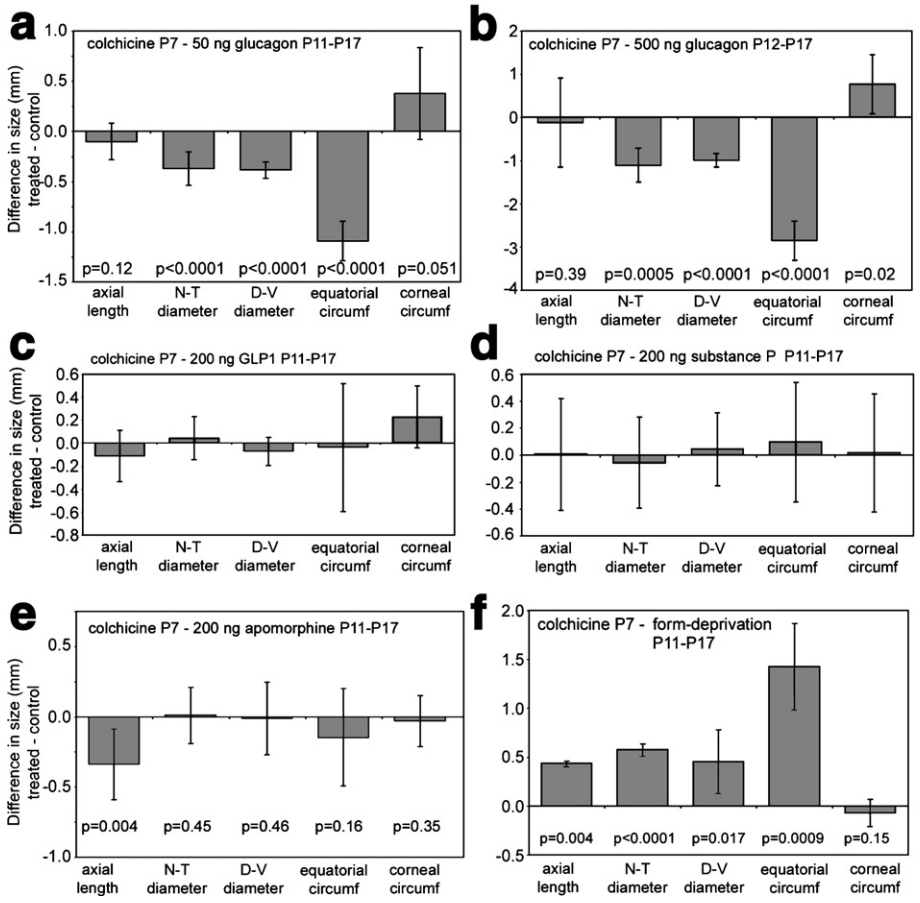

Fig. 6. Glucagon suppresses the equatorial eye growth in colchicine-treated eyes, whereas form-deprivation accelerates eye growth and GLP1, substance P and apomorphine have no effect. Both eyes were injected with 500 ng of colchicine at postnatal day (P7). Consecutive daily injections, beginning at P11 and ending at P17, were made into the left eyes and eyes were measured at P18 (a–e). The right eyes were injected with vehicle as a control. Alternatively, eyes were treated with colchicine at P7 and form-deprived from P11 through P17, and eyes measured at P18 (f). Compounds included 50 ng glucagon (a), 500 ng glucagon (b), 200 ng GLP1 (c), 200 ng substance P (d), or 200 ng apomorphine (e). Eye measurements included those for axial length, nasotemporal (N-T) equatorial diameter, dorsoventral (D-V) equatorial diameter, equatorial circumference, and corneal circumference. The histograms provide data for the mean ($n \geq 6$) and standard deviation. Significance of difference was determined by using a two-tailed, paired Student's *t*-test.

the control eyes, and this difference was highly significant (Fig. 7a). Exogenous glucagon had no significant affect upon corneal diameter (Fig. 7a).

To assess whether the antagonism of glucagon receptors influences ocular growth we made injections of a glucagon peptide antagonist (GPA), des-His^{1}, Phe^{6}, Glu^{9}-glucagon-NH_2. In addition, we applied a novel glucagon receptor antagonist, NNC 25-0926, which has been shown to effectively block the activity of glucagon in different biological systems (Lau et al., 2007; Qureshi et al., 2004; Rivera et al., 2007). Intraocular injections of 1000 ng GPA, 200 or 1000 ng of NNC 25-0926 were made from P6 through P11 and eyes measured at P12. The GPA did not significantly influence axial eye growth, whereas equatorial growth was uniformly increased across both nasotemporal and dorsoventral axes of the eye (Fig. 7b). The circumference of the equator of GPA-treated eyes was about 0.8 mm greater than that of contralateral control eyes (Fig. 7b). Interestingly, there was a significant increase in the corneal circumference in eyes treated with GPA (Fig. 7b). By contrast, 200 ng doses of NNC 25-0926 failed to significantly influence the size and shape of the eye (Fig. 7c). However, a 5-fold greater daily dose of NNC 25-0926 had significant affects upon ocular size and shape (Fig. 7d). We found that the axial length of NNC 25-0926-treated eyes was nearly 0.4 mm longer than that of contralateral control eyes (Fig. 7d). Although the nasotemporal equatorial diameter of NNC 25-0926-treated eyes was not significantly different from controls, the dorsoventral equatorial diameter was significantly increased and this was translated into a significant increase in circumference (Fig. 7d). Corneal circumference was significantly increased in eyes treated with 1000 ng of NNC 25-0926, similar to eyes treated with GPA (Fig. 7d).

We failed to find evidence that the glucagon receptor antagonists caused retinal damage or loss of bullwhip and mini-bullwhip cells and their terminals in the CMZ (data not shown). Consistent with our previous findings (Fischer et al., 2005), we found that the GPA stimulated the addition of new cells to the edge of the retina and non-pigmented epithelium (NPE) of the ciliary body. Similarly, we found that the NNC 25-0926 stimulated the addition of new cells to the edge of the retina and NPE of the ciliary body (data not shown). Taken together, these findings suggest that both the GPA and NNC 25-0926 do not enhance eye growth by damaging the retina, and that these

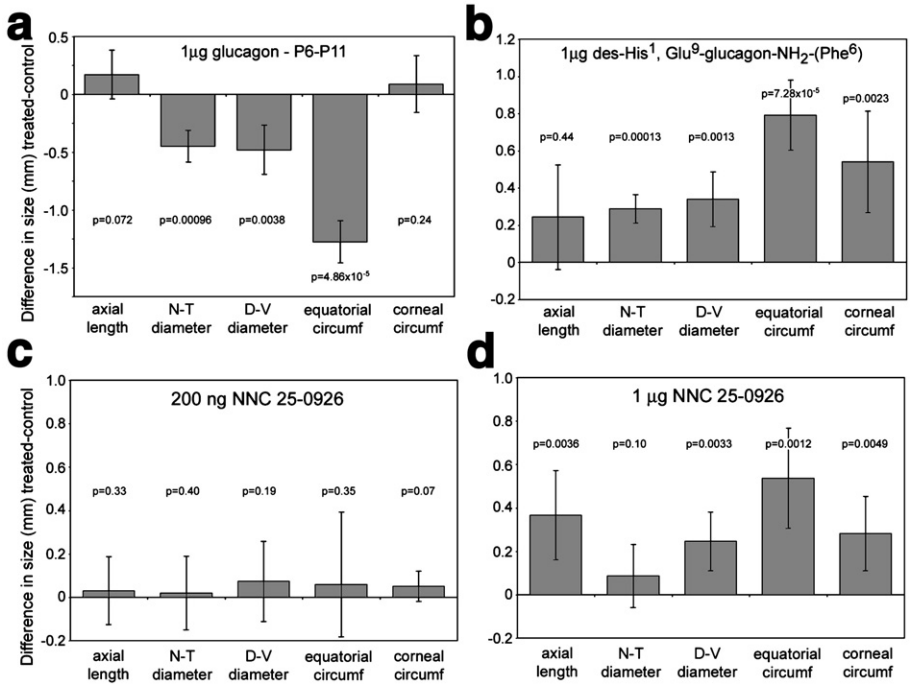

Fig. 7. Glucagon inhibits the equatorial growth of the eye, whereas glucagon receptor antagonists stimulate equatorial growth. The left eyes of chickens were injected daily with 1 µg of glucagon, 1 µg des-His[1], Phe[6], Glu[9]-glucagon-NH_2 or (200 ng or 1 µg) glucagon receptor antagonist (NNC 25-0926) from P6 through P11, right eyes received injections of saline, and eyes were measured at P12. The histograms provide data for the mean ($n=6$) and standard deviation. Significance of difference was determined by using a two-tailed, paired Student's *t*-test.

glucagon receptor antagonists stimulate the proliferation of CMZ progenitors in addition to acting at extra-retinal sites.

The bullwhip cells respond to visual stimuli that regulate rates of ocular growth

We have reported previously that the expression of the immediate early gene Egr1 is up-regulated by GACs in response to visual stimuli that slow rates of ocular growth (Fischer et al., 1999a). By contrast, the GACs down-regulate Egr1 in response to visual stimuli that accelerate rates of ocular growth. The growth-accelerating visual stimuli included form-deprivation (a light-diffusing goggle placed over the eye) and minus defocus (imposed by a −7 diopter lens fitted over the eye). The growth-slowing visual stimuli included plus defocus (imposed by a +7 diopter lens fitted over the eye) and unrestricted, normal vision after 5 days of form-deprivation. Here we sought to assess whether growth-regulating visual stimuli regulate the expression of Egr1 in the bullwhip cells.

In young chickens, form-deprivation is known to accelerate rates of ocular growth and render eyes myopic (reviewed by Wallman and Winawer, 2004). Here we confirmed that 5 days of form-deprivation caused eyes to grow significantly larger and more myopic than contralateral untreated eyes (data not shown). After 24 h of form-deprivation, the majority of GACs were negative for Egr1-immunoreactivity (data not shown), as described previously (Fischer et al., 1999a). Similarly, form-deprivation for 24 h caused a significant reduction in the percentage of bullwhip cells that expressed Egr1 (Figs. 8a–c and m). There was a significant reduction in the percentage of bullwhip cells that expressed Egr1 compared to the percentage observed in contra-lateral control retinas (Fig. 8m).

The "recovery" paradigm, in which a period of form-deprivation is followed by goggle-removal and unobstructed vision, is an alternative method of rendering the eyes myopically defocused. After 2 h of unrestricted vision following 5 days of form-deprivation, the majority of GACs were immunoreactive for Egr1 (Figs. 8d–f), consistent with previous reports (Bitzer and Schaeffel, 2002; Fischer et al., 1999a). Here, Egr1 was induced in more than 80% of the bullwhip cells after 5 days of form-deprivation followed by 2 h of unobstructed vision, compared to only about 25% after 5 days of continuously unobstructed vision (Figs. 8g–i and m). Similar to previous reports (Fischer et al., 1999a), we observed increased numbers of Egr1-positive nuclei in the bipolar cell layer of the INL in retinas with vision restored after 5 days of form-deprivation compared numbers seen in control and form-deprived retinas (compare Figs. 8a–c to d–i).

Receptors to glucagon and GLP1 are express by ocular tissues in equatorial regions of the eye

We next sought to assess whether glucagon receptors are expressed by the tissues that determine ocular shape and size. By using RT-PCR we detected mRNA for the glucagon receptor in the retina, choroid/RPE, and sclera (Fig. 9a). Similarly, we detected mRNA for the GLP1 receptor in the retina and choroid/RPE, and sclera (Fig. 9b). The PCR products were TOPO cloned and sequenced to verify the specificity of the PCR reactions. To identify the cell types in the ocular tissues that

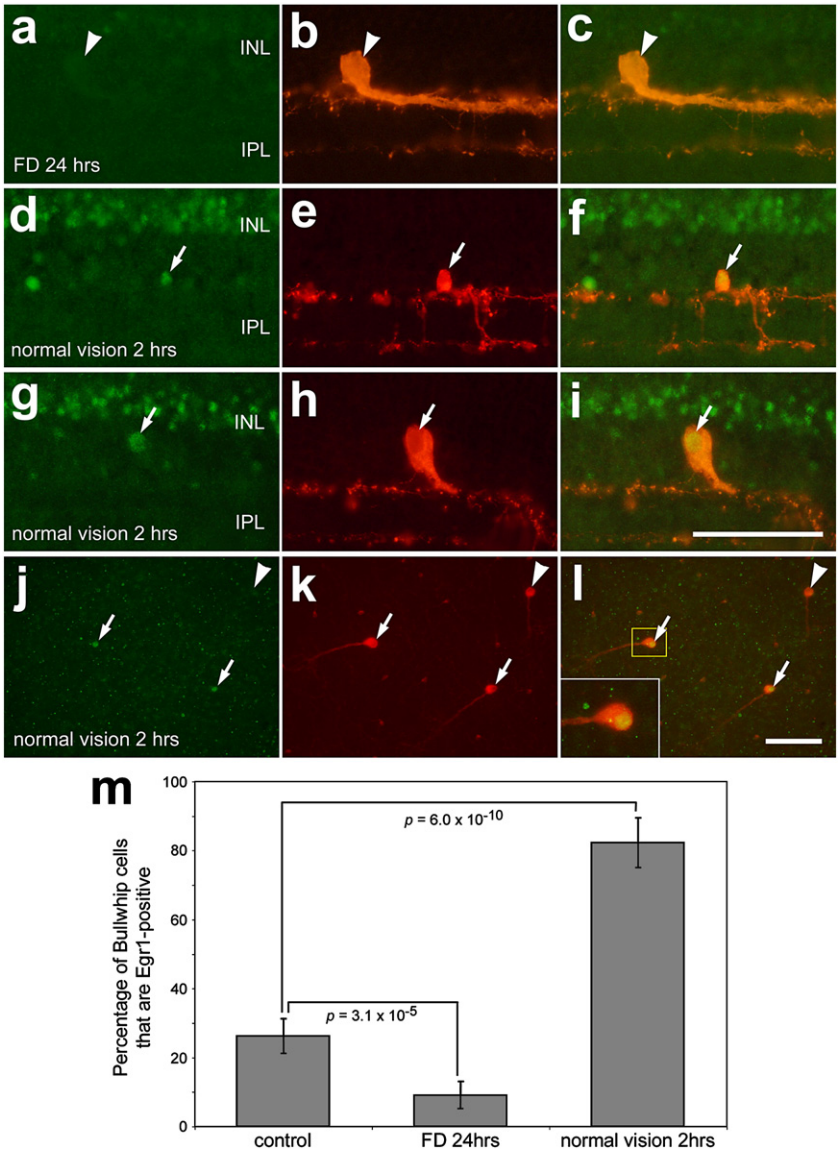

Fig. 8. Bullwhip cells respond to growth-slowing and growth-accelerating visual stimuli by up- or down-regulating Egr1, respectively. Vertical sections (a–i) and whole-mounts (j–l) of the retina where labeled with antibodies to Egr1 (green) and glucagon (red). Retinas were obtained from eyes that were form-deprived for 24 h (growth-accelerating) or form-deprived for 5 days followed by 2 h of normal vision (growth-slowing). Arrows indicate glucagon-positive amacrine cells or bullwhip cells with nuclei that are labeled for Egr1, and arrow-heads indicate bullwhip cells that are negative for Egr1. The calibration bar (50 μm) in panel i applies to panels a–i, and the bar in panel l applies to panels j–l. Panel m is a histogram illustrating the percentage of bullwhip cells that were immunoreactive for Egr1 in retinas from eyes that were untreated (control), form-deprived for 24 h, or form-deprived for 5 days followed by 2 h of normal vision. Significance of difference was determined by using ANOVA ($p<0.0001$) and a post-hoc Student's *t*-test; the *p* values are indicated in panel m. Bullwhip cells ($n=466$) were counted from at least 6 individuals from sections and whole-mount preparations of the retina. Abbreviations: INL—inner nuclear layer, IPL—inner plexiform layer, FD—form-deprived.

express the glucagon receptor we performed *in situ* hybridization (ISH). Consistent with previous reports (Feldkaemper et al., 2004; Fischer et al., 2005), we found ISH-signal for the glucagon receptor within the retina, concentrated in the ONL, proximal INL and GCL (Fig. 9c). The ISH-signal was present in most photoreceptors, cells in the amacrine layers of the INL, and cells in the GCL. At the peripheral edge of the retina, mRNA for the glucagon receptor was detected within the CMZ, the non-pigmented epithelium (NPE) of the ciliary body, and in the ONL adjacent to the CMZ (Fig. 9d). The signal for the glucagon receptor in the NPE extended through the pars plana and into the pars plicata (Fig. 9e). Relatively little signal for the glucagon receptor was observed in the RPE and choroid. By comparison, we observed ISH-labeling in the sclera. There was relatively weak labeling in the cartilaginous sclera, whereas labeling of greater intensity was observed in the fibrous sclera (Figs. 9f and g). This labeling was present in the sclera that lies behind peripheral (Fig. 9f) and central regions of the retina (Supplemental Fig. 4a), and at the anterior edge of the cartilaginous sclera at the equator of the eye which lies near the peripheral edge of the retina (Fig. 9g). Further, we observed robust signal for the glucagon receptor in stromal and epithelial compartments of the corneal limbus, whereas weak ISH-signal for the glucagon receptor was seen in the corneal epithelium and

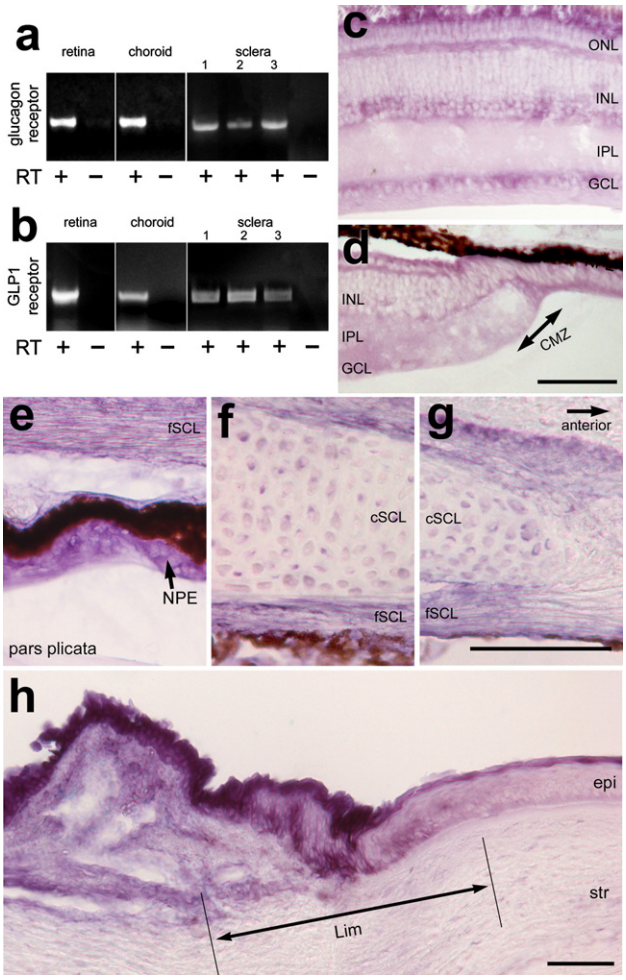

Fig. 9. mRNA for glucagon receptor and GLP1 receptor are expressed by the retina, choroid/RPE, and sclera in equatorial regions of the eye. mRNA was detected by using reverse-transcriptase (RT) to generate pools of cDNA and PCR with sequence-specific primers to glucagon receptor and GLP1 receptor. Total RNA was extracted and pooled from P7 retina and from choroid/RPE, whereas RNA samples from sclera were keep separate for each individual. mRNA was reverse transcribed into cDNA by using SuperScript III™ reverse transcriptase (RT+), while control samples were reacted without enzyme (RT−). *In situ* hybridization for mRNA coding for the glucagon receptor was performed on sections of the retina (c), CMZ (d), pars plicata (e), peripheral sclera (f), anterior edge of the cartilaginous sclera (g), and corneal limbus (h). The calibration bar (50 μm) in panel d applies to panels c and d, the bar in panel g applies to panels e–g, and the bar in panel h applies to panel h alone. Abbreviations: ONL—outer nuclear layer, INL—inner nuclear layer, IPL—inner plexiform layer, GCL—ganglion cell layer, CMZ—circumferential marginal zone, NPE—non-pigmented epithelium, fSCL—fibrous sclera, cSCL—cartilaginous sclera, Lim—corneal limbus, epi—corneal epithelium, str—corneal stroma.

central regions of the stroma (Fig. 9h). Sense riboprobes failed to produce significant levels of labeling in all ocular tissues (Supplemental Figs. 4b–e).

Discussion

Our findings indicate that the shape and size of a complex organ, the eye, can be influenced by a relatively minor population (<1000 cells) of intrinsic retinal neurons, the bullwhip and mini-bullwhip cells. We find that colchicine destroys the vast majority of the glucagonergic bullwhip and mini-bullwhip cells, as well as some retinal cells in far-peripheral and dorsal regions of the retina, whereas the glucagon-containing amacrine cells are unaffected. The colchicine-treated eyes grow excessively around the equator, whereas corneal growth is suppressed and axial eye length remains unaffected. Regardless of any colchicine-mediated side-effects, exogenous glucagon completely inhibits the effects of colchicine-treatment on eye growth, whereas GLP1, substance P and a dopamine receptor agonist had no effect. This argues strongly that the depletion of glucagon, due to the colchicine-mediated destruction of bullwhip and mini-bullwhip, causes excessive equatorial eye growth in colchicine-treated eyes.

We propose that glucagon, which is released from the terminals of the bullwhip and mini-bullwhip cells at the peripheral edge of the retina, acts to slow equatorial eye growth. The overall size and shape of the eye are determined by the growth and remodeling of the sclera, the cartilaginous sheath of the eye (reviewed by Rada et al., 1994; Wallman and Winawer, 2004). We found that the depletion of glucagon-releasing terminals from the edge of the retina and the application of glucagon receptor antagonists caused excessive growth around the equator of the eye. Consistent with these findings, intraocular injections of glucagon suppressed equatorial eye growth in normal eyes and in eyes lacking bullwhip cells. Our data indicate that axial and equatorial eye growth can occur independently and are regulated by different retinal cell types. Colchicine-treatment at P7 did not ablate the CAGs, and axial eye length was normal, whereas equatorial growth was increased. In addition, form-deprivation caused axial and equatorial ocular enlargement of colchicine-treated eyes, where the bullwhip and mini-bullwhip cells were ablated and the CAGs remained intact. These findings suggest that the retinal circuitry and cells, namely the CAGs, that permit accelerated axial eye growth in response to degraded image contrast are unaffected by colchicine-treatment at P7. Thus, we propose that axial eye length is regulated by the CAGs and equatorial growth is regulated by the bullwhip and mini-bullwhip cells.

Consistent with the hypothesis that retinal glucagon regulates the growth of the ocular equator and cornea, we found that glucagon receptors are widely expressed by anterior ocular tissues. Glucagon receptor mRNA was detected at the peripheral edge of the retina, in the NPE of the ciliary body, the sclera and the corneal limbus. We propose that these glucagon receptors are normally activated by glucagon that is produced by retinal neurons. The only sources of glucagon peptide within the eye are the GACs, bullwhip cells and mini-bullwhip cells. Nevertheless, it is possible that systemic glucagon, produced by pancreatic cells, acts at receptors in the eye to influence overall growth. However, it seems unlikely that pancreas-derived glucagon participates in vision-guided ocular growth. Thus, we propose that glucagon peptide that is released from the terminals of the bullwhip and mini-bullwhip cells at the peripheral edge of the retina could act on the fibrous or cartilaginous sclera to influence equatorial eye growth, as well as corneal growth. See Fig. 10 for a schematic summary.

Although reducing glucagon-signaling significantly increased equatorial scleral growth, at the same time corneal growth (cir-

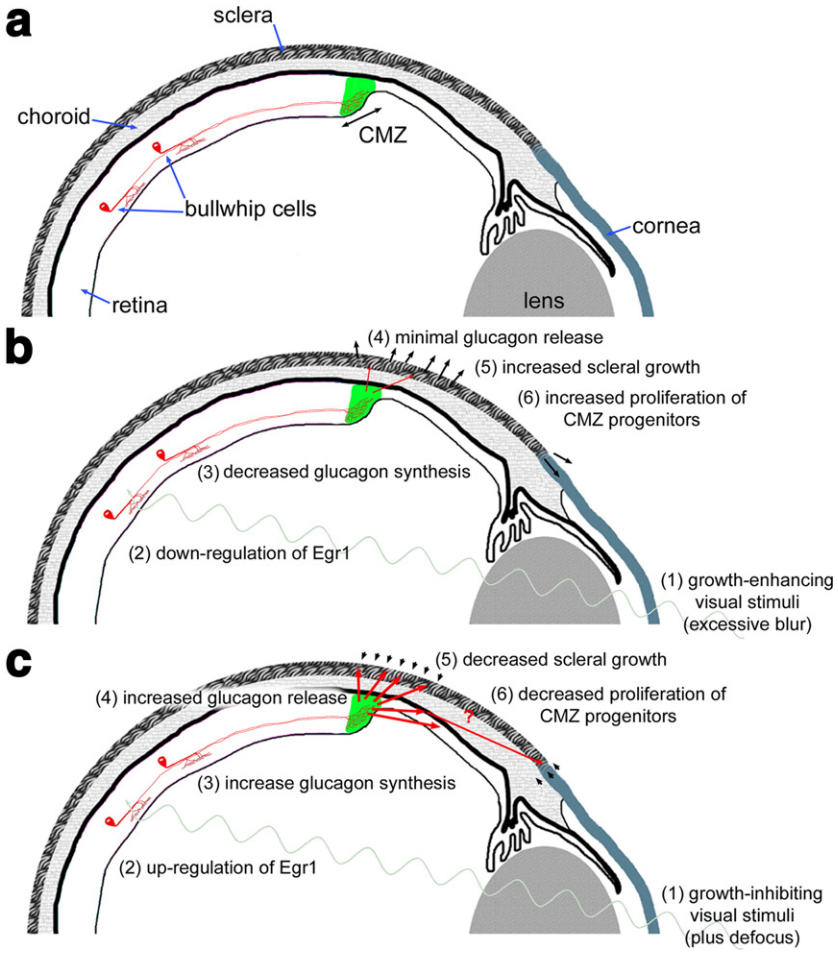

Fig. 10. Schematic diagram describing the proposed progression of events by which visual stimuli, glucagon and bullwhip cells regulate the equatorial growth of the eye and cornea. The red arrows indicate the release and dissemination of glucagon peptide from terminals that are clustered in the CMZ. The black arrows indicate the relative direction of change in eye shape in response to growth-guiding visual stimuli and glucagon release. Panel a illustrates the relative position of the bullwhip cells and glucagon-releasing terminal in the CMZ to the equatorial sclera and cornea. Panel b summarizes the effects of growth-enhancing visual stimuli on the bullwhip cells, glucagon release, and eye growth. Panel c summarizes the effects of growth-inhibiting visual stimuli on the bullwhip cells, glucagon release, and eye growth.

cumference) was decreased. Therefore, our data indicate that corneal and scleral growth are regulated independently, at least in part. The mechanisms underlying the reduced corneal growth in colchicine-treated eyes remain uncertain. It is possible that intravitreal delivery of colchicine destroyed the corneal limbal stem cells, which in turn suppressed normal corneal growth. Alternatively, glucagon that is released from the terminals of the bullwhip and mini-bullwhip cells at the periphery of the retina may normally stimulate corneal growth. This would require that glucagon peptide diffuses from the peripheral edge of the retina out to the corneal limbus. In support of this possibility, we found that intravitreal delivery of glucagon peptide blocked colchicine-mediated decreases in corneal circumference. However, glucagon had no affect on corneal circumference in eyes with undamaged retinas, and the glucagon receptor antagonists increased corneal diameter in eyes with undamaged retinas. It remains uncertain how corneal circumference is differentially regulated by glucagon receptor agonist and antagonists in normal and colchicine-treated eyes. We propose that there may be coordination of retina-derived signals with the growth of the cornea during postnatal development. Similarly, the embryonic eye relies heavily on reciprocal interactions of the optic vesicle/cup (prospective retina) with the overlying ectoderm (prospective lens and cornea) for proper ocular development (reviewed by Chow and Lang, 2001).

The reported effects of glucagon and receptor antagonists on ocular growth have been inconsistent. Vessey and colleagues have reported that injections of glucagon or glucagon antagonist (des-Phe^6-glucagon-NH_2) do not influence the normal axial growth of eyes with unrestricted vision (Vessey et al., 2005a). The authors proposed that the glucagon antagonist is not entirely effective at chicken glucagon receptors and that the glucagon peptide did not influence ocular growth because the endogenous growth-suppressing signals are already near or at saturation. The study by Vessey and colleagues used 5 consecutive daily intraocular injections of nearly 700 ng of glucagon or antagonist to open eyes, but failed to find significant changes in refractive error or axial length. It remains possible that glucagon and glucagon receptor antagonist influenced equatorial eye growth, but these changes went unmeasured (W.K. Stell, personal communication). We found that 6 consecutive daily intraocular injections of 1000 ng glucagon sup-

pressed equatorial eye growth, whereas axial length was not affected. In line with these observations, we found that 5 consecutive daily injections of 1000 ng des-His[1], Phe[6], Glu[9]-glucagon-NH_2 failed to influence axial eye growth, whereas equatorial eye growth was increased. Similarly, a small molecular inhibitor of the glucagon receptor NNC 25-0926 caused increases in axial length and equatorial diameter. The growth enhancing effects of NNC 25-0926 were pronounced across the dorsoventral equatorial axis. This lipophilic compound was injected consistently into the dorsal quadrant of the eye and, thus, may have preferentially influenced the growth of dorsal equatorial regions of the eye. The subtle differences in the effects of the glucagon receptor antagonists upon axial and equatorial growth may reflect differences in the efficacy and/or ability of these compounds to diffuse within the eye and avoid clearance and degradation.

Similar to the GACs, the bullwhip cells respond to growth-regulating visual stimuli. Form-deprivation prevents clear images from being projected onto the retina; insufficient image contrast fails to activate the retinal circuitry to halt eye growth (reviewed by Wallman and Winawer, 2004). In the postnatal chicken, the deprivation of clear vision or minus defocus causes a down-regulation in the expression of Egr1 in the GACs (Fischer et al., 1999a). Conversely, growth-halting visual stimuli such as plus-defocus or restoration of clear vision after form-deprivation stimulate the expression of Egr1 in the GACs (Fischer et al., 1999a). We report here that the bullwhip cells up- or down-regulate Egr1 in response to growth-slowing or growth-accelerating visual stimuli, respectively. However, it should be noted that the bullwhip cells are a distinctly different type of cell compared to the GACs; with different morphologies, neurotransmitters, and immunohistochemical profiles (Fischer et al., 2006). Despite these differences, it seems likely that both the bullwhip cells and GACs participate in the retinal circuitry that detects image defocus to regulate eye size and shape. We propose that growth-regulating visual stimuli, acting through intermediate retinal circuitry, regulate the activity of bullwhip cells and thereby the rate of release of glucagon at the retinal margin. This results in either increased glucagon release, which retards equatorial eye growth; or decreased glucagon release, which allows equatorial eye growth to continue unimpeded. Similarly, reduced release of glucagon from the terminals of the bullwhip and mini-bullwhip cells may allow for increased proliferation of progenitors in the CMZ (Fischer et al., 2005). Taken together, our findings may explain why CMZ progenitors proliferate at increased rates in form-deprived eyes (Fischer and Reh, 2000). We propose that bullwhip cells are relatively inactive during form-deprivation, and consequently the rate of glucagon release is relatively low. Therefore, in form-deprived eyes the proliferation-suppressing influence of glucagon is relieved, and the progenitors proliferate at increased rates. Vision-regulated release of glucagon from the terminals of the bullwhip and mini-bullwhip may coordinate the growth of the retina, equatorial growth of the sclera, and circumferential growth of the cornea. This proposed mechanism of regulating retinal and equatorial eye growth is illustrated in Fig. 10.

Colchicine treatment at P7 caused some cell death within the CMZ, indicating that colchicine destroyed some of the proliferating progenitors. Colchicine is known to cause M-phase arrest, and this, in turn, can lead to cell death (reviewed by Downing, 2000). The loss of CMZ progenitors may have, secondarily, caused the loss of bullwhip cells. We have suggested elsewhere that the bullwhip cells require trophic support from target cells to survive (Fischer et al., 2005, 2006, 2007). Thus, it is possible that colchicine-mediated death of CMZ progenitors depletes the bullwhip cells of trophic support, and thereby causes the death of the bullwhip cells. Additionally, colchicine may disrupt the microtubules in the axons of the bullwhip cells, thereby preventing retrograde transport of trophic signals back to the cell bodies. Colchicine is known to prevent the retrograde axonal transport of trophic cues and attenuate the survival of many different types of projection neurons (reviewed by Avila, 1992).

The GACs, dopaminergic amacrine cells, and the majority of ganglion cells are sensitive to colchicine shortly after hatching, before P2. However, these cell types become tolerant to colchicine-treatment shortly after early stages of postnatal retinal development, with the exception of the ganglion cells in far-peripheral and dorsal regions of the retina. It is likely that the ganglion cells that remain sensitive to colchicine at P7 are relatively immature. Ganglion cells are known to be first generated in central–ventral regions of the retina, whereas the ganglion cells in dorsal and peripheral regions of retina are generated about 3 days later (Kahn, 1973, 1974; Prada et al., 1991). Similarly, far-peripheral regions of the chicken retina are generated late during embryonic development, between E12 and E16, with nasal regions being generated before temporal regions (Ghai et al., 2008). We propose that the immaturity of neurons in far temporal regions of the retina renders these cells more susceptible to colchicine-induced cell death compared to the more mature cells in far nasal regions of the retina. Sensitivity to colchicine may be manifested through active remodeling of microtubules, which are prevalent in maturing neurons (Black, 1994; Kobayashi and Mundel, 1998).

Conclusions

Despite any colchicine-mediated side-effects upon retinal cells in addition to the bullwhip and mini-bullwhip cells, replacement of glucagon peptide into treated eyes completely prevented changes in eye shape and size that resulted from colchicine-treatment. In colchicine-treated eyes, glucagon peptide is depleted because of the destruction of bullwhip and mini-bullwhip cells and their terminals at the far-peripheral edge of the retina. Because the terminals of bullwhip and mini-bullwhip cells are concentrated near the equator of the eye, it seems logical that one function of the glucagon that they release is to restrict scleral growth at the equator. We conclude that the bullwhip and mini-bullwhip cells, despite their modest numbers, have a profound influence on the shape and size of the eye. We propose that the bullwhip and mini-bullwhip cells, like the conventional glucagon-expressing amacrine cells, are part of the retinal circuitry that detects image defocus; these

defocus cues are used by the bullwhip and mini-bullwhip cells to precisely regulate the equatorial growth of the eye, the retina and, possibly, corneal circumference.

Acknowledgments

We thank Drs. Heithem M. El-Hodiri, Don Mutti, Stuart Mangel and William K. Stell for comments that contributed to the final form of this paper. We thank Drs. Virginia Lee, Paul Linser, and Steve McLoon for providing antibodies to neurofilament, 2M6, and RA4, respectively. Expert technical assistance with confocal microscopy was provided at the Campus Microscopy and Imaging Facility at The Ohio State University. The tyrosine hydroxylase antibody, developed by Drs. Le Douarin and Ziller, was obtained from the Developmental Studies Hybridoma Bank developed under auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported by a grant (#0413795) from the National Science Foundation awarded to AJF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.02.023.

References

Avila, J., 1992. Microtubule functions. Life Sci. 50, 327–334.

Bitzer, M., Schaeffel, F., 2002. Defocus-induced changes in ZENK expression in the chicken retina. Invest. Ophthalmol. Visual Sci. 43, 246–252.

Black, M.M., 1994. Microtubule transport and assembly cooperate to generate the microtubule array of growing axons. Prog. Brain. Res. 102, 61–77.

Chow, R.L., Lang, R.A., 2001. Early eye development in vertebrates. Annu. Rev. Cell Dev. Biol. 17, 255–296.

Del Rio-Tsonis, K., Tsonis, P.A., 2003. Eye regeneration at the molecular age. Dev. Dyn. 226, 211–224.

Downing, K.H., 2000. Structural basis for the interaction of tubulin with proteins and drugs that affect microtubule dynamics. Annu. Rev. Cell Dev. Biol. 16, 89–111.

Ehrlich, D., Keyser, K.T., Karten, H.J., 1987. Distribution of substance P-like immunoreactive retinal ganglion cells and their pattern of termination in the optic tectum of chick (*Gallus gallus*). J. Comp. Neurol. 266, 220–233.

Feldkaemper, M.P., Schaeffel, F., 2002. Evidence for a potential role of glucagon during eye growth regulation in chicks. Vis. Neurosci. 19, 755–766.

Feldkaemper, M.P., Burkhardt, E., Schaeffel, F., 2004. Localization and regulation of glucagon receptors in the chick eye and preproglucagon and glucagon receptor expression in the mouse eye. Exp. Eye Res. 79, 321–329.

Fischer, A.J., 2005. Neural regeneration in the chick retina. Prog. Retin. Eye Res. 24, 161–182.

Fischer, A.J., Reh, T.A., 2000. Identification of a proliferating marginal zone of retinal progenitors in postnatal chickens. Dev. Biol. 220, 197–210.

Fischer, A.J., Reh, T.A., 2003. Growth factors induce neurogenesis in the ciliary body. Dev. Biol. 259, 225–240.

Fischer, A.J., Stell, W.K., 1999. Nitric oxide synthase-containing cells in the retina, pigmented epithelium, choroid, and sclera of the chick eye. J. Comp. Neurol. 405, 1–14.

Fischer, A.J., Miethke, P., Morgan, I.G., Stell, W.K., 1998a. Cholinergic amacrine cells are not required for the progression and atropine-mediated suppression of form-deprivation myopia. Brain Res. 794, 48–60.

Fischer, A.J., Seltner, R.L., Poon, J., Stell, W.K., 1998b. Immunocytochemical characterization of quisqualic acid- and *N*-methyl-D-aspartate-induced excitotoxicity in the retina of chicks. J. Comp. Neurol. 393, 1–15.

Fischer, A.J., Seltner, R.L., Stell, W.K., 1998c. Opiate and *N*-methyl-D-aspartate receptors in form-deprivation myopia. Vis. Neurosci. 15, 1089–1096.

Fischer, A.J., McGuire, J.J., Schaeffel, F., Stell, W.K., 1999a. Light- and focus-dependent expression of the transcription factor ZENK in the chick retina. Nat. Neurosci. 2, 706–712.

Fischer, A.J., Morgan, I.G., Stell, W.K., 1999b. Colchicine causes excessive ocular growth and myopia in chicks. Vis. Res. 39, 685–697.

Fischer, A.J., Dierks, B.D., Reh, T.A., 2002. Exogenous growth factors induce the production of ganglion cells at the retinal margin. Development 129, 2283–2291.

Fischer, A.J., Omar, G., Eubanks, J., McGuire, C.R., Dierks, B.D., Reh, T.A., 2004. Different aspects of gliosis in retinal Muller glia can be induced by CNTF, insulin and FGF2 in the absence of damage. Mol. Vis. 10, 973–986.

Fischer, A.J., Omar, G., Walton, N.A., Verrill, T.A., Unson, C.G., 2005. Glucagon-expressing neurons within the retina regulate the proliferation of neural progenitors in the circumferential marginal zone of the avian eye. J. Neurosci. 25, 10157–10166.

Fischer, A.J., Skorupa, D., Schonberg, D.L., Walton, N.A., 2006. Characterization of glucagon-expressing neurons in the chicken retina. J. Comp. Neurol. 496, 479–494.

Fischer, A.J., Stanke, J.J., Ghai, K., Scott, M., Omar, G., 2007. Development of bullwhip neurons in the embryonic chicken retina. J. Comp. Neurol. 503, 538–549.

Ghai, K., Stanke, J.J., Fischer, A.J., 2008. Patterning of the circumferential marginal zone of progenitors in the chicken retina. Brain Res. 1192, 76–89.

Kahn, A.J., 1973. Ganglion cell formation in the chick neural retina. Brain Res. 63, 285–290.

Kahn, A.J., 1974. An autoradiographic analysis of the time of appearance of neurons in the developing chick neural retina. Dev. Biol. 38, 30–40.

Kobayashi, N., Mundel, P., 1998. A role of microtubules during the formation of cell processes in neuronal and non-neuronal cells. Cell Tissue Res. 291, 163–174.

Lau, J., Behrens, C., Sidelmann, U.G., Knudsen, L.B., Lundt, B., Sams, C., Ynddal, L., Brand, C.L., Pridal, L., Ling, A., Kiel, D., Plewe, M., Shi, S., Madsen, P., 2007. New beta-alanine derivatives are orally available glucagon receptor antagonists. J. Med. Chem. 50, 113–128.

Morgan, I.G., 1981. Intraocular colchicine selectively destroys immature ganglion cells in chicken retina. Neurosci. Lett. 24, 255–260.

Moshiri, A., McGuire, C.R., Reh, T.A., 2005. Sonic hedgehog regulates proliferation of the retinal ciliary marginal zone in posthatch chicks. Dev. Dyn. 233, 66–75.

Otteson, D.C., Hitchcock, P.F., 2003. Stem cells in the teleost retina: persistent neurogenesis and injury-induced regeneration. Vis. Res. 43, 927–936.

Prada, C., Puga, J., Perez-Mendez, L., Lopez, R., Ramirez, G., 1991. Spatial and temporal patterns of neurogenesis in the chick retina. Eur. J. Neurosci. 3, 559–569.

Qureshi, S.A., Rios Candelore, M., Xie, D., Yang, X., Tota, L.M., Ding, V.D., Li, Z., Bansal, A., Miller, C., Cohen, S.M., Jiang, G., Brady, E., Saperstein, R., Duffy, J.L., Tata, J.R., Chapman, K.T., Moller, D.E., Zhang, B.B., 2004. A novel glucagon receptor antagonist inhibits glucagon-mediated biological effects. Diabetes 53, 3267–3273.

Rada, J.A., Matthews, A.L., Brenza, H., 1994. Regional proteoglycan synthesis in the sclera of experimentally myopic chicks. Exp. Eye Res. 59, 747–760.

Rada, J.A., Shelton, S., Norton, T.T., 2006. The sclera and myopia. Exp. Eye Res. 82, 185–200.

Raymond, P.A., Hitchcock, P.F., 1997. Retinal regeneration: common principles but a diversity of mechanisms. Adv. Neurol. 72, 171–184.

Raymond, P.A., Hitchcock, P.F., 2000. How the neural retina regenerates. Results Probl. Cell Differ. 31, 197–218.

Reh, T.A., Levine, E.M., 1998. Multipotential stem cells and progenitors in the vertebrate retina. J. Neurobiol. 36, 206–220.

Rivera, N., Everett-Grueter, C.A., Edgerton, D.S., Rodewald, T., Neal, D.W., Nishimura, E., Larsen, M.O., Jacobsen, L.O., Kristensen, K., Brand, C.L.,

Cherrington, A.D., 2007. A novel glucagon receptor antagonist, NNC 25-0926, blunts hepatic glucose production in the conscious dog. J. Pharmacol. Exp. Ther. 321, 743–752.

Rohrer, B., Spira, A.W., Stell, W.K., 1993. Apomorphine blocks form-deprivation myopia in chickens by a dopamine D2-receptor mechanism acting in retina or pigmented epithelium. Vis. Neurosci. 10, 447–453.

Stone, R.A., Lin, T., Laties, A.M., Iuvone, P.M., 1989. Retinal dopamine and form-deprivation myopia. Proc. Natl. Acad. Sci. U. S. A. 86, 704–706.

Vessey, K.A., Lencses, K.A., Rushforth, D.A., Hruby, V.J., Stell, W.K., 2005a. Glucagon receptor agonists and antagonists affect the growth of the chick eye: a role for glucagonergic regulation of emmetropization? Invest. Ophthalmol. Visual Sci. 46, 3922–3931.

Vessey, K.A., Rushforth, D.A., Stell, W.K., 2005b. Glucagon- and secretin-related peptides differentially alter ocular growth and the development of form-deprivation myopia in chicks. Invest. Ophthalmol. Visual Sci. 46, 3932–3942.

Wallman, J., Winawer, J., 2004. Homeostasis of eye growth and the question of myopia. Neuron 43, 447–468.